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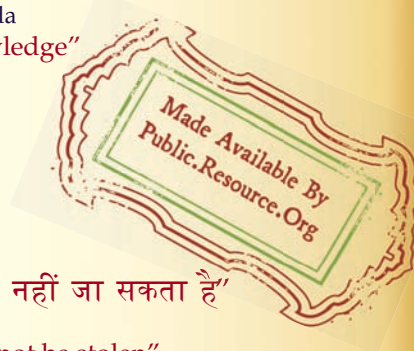
IS 13002 (1990): Method for enumeration of living aerobic micro-organisms of bull semen [FAD 5: Livestock Feeds, Equipment and Systems]



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Bhartrhari—Nitiśatakam

“Knowledge is such a treasure which cannot be stolen”

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IS 13002 : 1990
(Reaffirmed 2000)

Indian Standard

**METHOD FOR
ENUMERATION OF LIVING AEROBIC
MICRO-ORGANISMS IN BULL SEMEN**

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BUREAU OF INDIAN STANDARDS
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NEW DELHI 110002

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FOREWORD

This Indian Standard was adopted by the Bureau of Indian Standards on 25 June 1990, after the draft finalized by the Animal Reproduction Equipment and Systems Sectional Committee had been approved by the Food and Agriculture Division Council.

Frozen semen is being increasingly used for improvement of the quality of livestock in the country. The frozen semen for artificial insemination should be of better quality in order to achieve satisfactory results. This necessitates the semen to be free from various micro-organisms. The method for enumeration of living aerobic micro-organism has been given in this standard.

In preparation of this standard, assistance has been derived from ISO/DIS 8607 'Artificial insemination of animals — Bull semen — Enumeration of living aerobic micro-organism', issued by International Organization for Standardization.

In reporting the result of a test made in accordance with this standard, if the final value, observed or calculated is to be rounded off, it shall be done in accordance with IS 2 : 1960 'Rules for rounding off numerical values (*revised*)'.

Indian Standard

METHOD FOR ENUMERATION OF LIVING AEROBIC MICRO-ORGANISMS IN BULL SEMEN

1 SCOPE

This standard specifies method for the enumeration of living aerobic micro-organisms present in bull semen.

2 DEFINITION

For the purpose of this standard, the following definition shall apply.

2.1 Living Aerobic Micro-organisms

Bacteria, yeasts and moulds which grow aerobically at 37°C under the conditions described in this standard.

3 PRINCIPLE

3.1 Deep inoculation of a specific culture medium poured in two petri dishes with a definite quantity of test sample diluted to the necessary degree.

3.2 Aerobic incubation of the two plates for 72 h at $37 \pm 1^\circ\text{C}$.

3.3 Calculation of the number of micro-organisms per millilitre of test sample from the number of colonies obtained.

4 DILUENT AND CULTURE MEDIUM

4.1 Basic Materials

In order to improve the reproducibility of the results, it is recommended that, for the preparation of the diluent and culture medium, dehydrated basic components or a completely dehydrated medium be used. Similarly, commercially prepared reagents may also be used. The manufacture's instructions shall be rigorously followed.

4.1.1 The chemical products used for the preparation of the culture medium shall be of recognized analytical quality.

4.1.2 The water used shall be double-glass distilled and deionized, free from substances that might inhibit growth of micro-organisms under the test conditions.

4.1.3 Measurements of pH shall be made using a pH meter with measurements being referred

to a temperature of 25°C.

4.1.4 If the prepared culture media are not used immediately, they shall unless otherwise stated, be stored in the dark at 0 and 5°C in conditions which do not produce any change in their composition.

4.2 Diluent

Use sterile physiological saline solution, the sodium chloride (NaCl) content of which is 0.85 percent (*m/m*).

4.3 Agar Medium

Various components of the medium and their quantities shall be as given in Table 1.

Table 1 Components of the Agar Medium

Sl No.	Component	Quantity
(1)	(2)	(3)
i)	Meat extract	10.0 g
ii)	Anhydrous D-glucose ($\text{C}_6\text{H}_{12}\text{O}_6$)	1.0 g
iii)	Dehydrated yeast extract	2.5 g
iv)	Bacto-peptone	3.0 g
v)	Sodium chloride (NaCl)	2.0 g
vi)	Disodium hydrogen ortho-phosphate dodecahydrate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$)	2.0 g
vii)	Gelatine	10.0 g
viii)	Agar in powder or flake form	13.0 to 15.0 g*
ix)	Water	1.0 l

*According to the manufacturer's instructions.

4.3.1 Dissolve the components or the dehydrated complete medium in water by boiling. Adjust the pH, if necessary (checking with a pH meter), so that after sterilization it is 7.4 ± 0.1 .

4.3.2 Dispense the media into bottles of 500 ml capacity up to half its volume. Sterilize in an autoclave at $121 \pm 1^\circ\text{C}$ for 20 minutes.

4.3.3 If the medium is to be used immediately, cool it to $45 \pm 0.5^\circ\text{C}$ in the water-bath and complete with 10 percent inactivated cattle serum sterilized by ultrafiltration or tyndallization. Otherwise before beginning the microbiological examination, completely melt the

medium in a boiling water-bath, cool to $45 \pm 0.5^\circ\text{C}$ in the water-bath then add 10 percent inactivated and sterilized cattle serum to it.

5 APPARATUS AND GLASSWARE

Besides usual microbiological laboratory equipment the following shall be used.

5.1 Apparatus for dry sterilization (oven) or wet sterilization (autoclave) operating either separately or as part of an apparatus for preparing and distributing media.

5.2 Incubator capable of being maintained at $37 \pm 1^\circ\text{C}$.

5.3 Test-tubes of diameter 16 mm and length 160 mm, or flasks of appropriate capacity.

5.4 Petri dishes of diameter 90 to 100 mm.

5.5 Pipettes (not blow-out pipettes) having a nominal capacity of 1 ml, graduated in divisions of 0.1 ml.

5.6 Automatic dispenser pipette.

5.7 pH-meter (Electric)

Accurate to ± 0.1 pH unit at 25°C .

5.8 Water-bath

Capable of being maintained at $37 \pm 0.5^\circ\text{C}$ and another at $45 \pm 0.5^\circ\text{C}$.

5.9 Colony counting equipment consisting of an illuminated base with a dark background fitted with a magnifying lens to be used at a magnification of 1.5 diameters, and a mechanical or electronic digital counter.

6 STERILIZATION

6.1 Apparatus that will come into contact with the diluent, the sample or the dilutions, except for apparatus that is supplied sterile (plastic bags, plastic pipettes, etc) shall be sterilized by one of the following methods:

- By keeping at 170 to 175°C for not less than 1 h in an oven, and
- By being kept at $121 \pm 1^\circ\text{C}$ for not less than 20 min in an autoclave.

7 COLLECTION OF SAMPLES

7.1 Take out randomly the necessary number of doses of frozen semen from any type of packaging so that the quantity of semen sample is 1.0 ml (minimum). The sample taken from the container that serves for 28 days control

should be of ejaculates from the same bull collected on one day and bears the same production number.

8 PREPARATION OF TEST SAMPLE

8.1 The semen samples shall be stored in liquid nitrogen until microbiological examination. For sampling they shall be taken out of the large storage container and transferred to a small laboratory container.

8.2 Deep-frozen semen samples should be thawed in a water-bath at 37°C for 3 minutes. Until dilution they may be kept in a refrigerator at 4°C for no longer than 1 h.

9 PROCEDURE

9.1 Test Portion, Initial Suspension and Dilution

Use 1 ml of semen for dilution with the diluent (*see 4.2*).

9.1.1 If the diluted semen contains the usual quantity of antibiotics, that is, 100 000 i.u. penicillin and 0.1 g streptomycin or other broad spectrum antibiotics to 100 ml diluent, a 1-10 000 final dilution should be used.

9.1.2 If the quantity of antibiotics differs from that mentioned in **9.1.1**, use a dilution such that the maximum quantity of penicillin is not more than 0.1 i.u. and that of broad spectrum antibiotics not more than 0.1 ug/ml. A higher antibiotics concentration may inhibit growth of the micro-organisms and produce false results.

9.1.3 If the deep-frozen semen does not contain any antibiotics, it can be used without dilution.

9.2 Inoculation and Incubation

9.2.1 Take two sterile petri dishes. Transfer, by means of a sterile pipette 1 ml of semen from the final dilution (*see 9.1*) to each dish.

9.2.2 Pour about 15 ml of the agar medium (*see 4.3*), at $45 \pm 0.5^\circ\text{C}$, into each petri dish. The time between the end of dilution and the moment when the medium is poured into the dishes shall not exceed 15 min.

9.2.2.1 Carefully mix the inoculum with the medium and allow it to solidify by leaving the petri dishes to stand on a cool horizontal surface.

9.2.3 Incubation

Invert the prepared dishes and place, them in the incubator maintained at $37 \pm 1^\circ\text{C}$ for 72 h.

9.3 Interpretation

After the incubation period, count (by means of the colony counting equipment) the colonies in both dishes. Count only those well-distinguishable colonies which have grown within the medium. Ignore colonies which have grown on the surface of the medium. Do not count on those plates on which half or more of the area joining colonies have developed.

10 EXPRESSION OF RESULTS

10.1 If one or both dishes corresponding to the same dilution contain between 5 and 300 colonies, calculate the arithmetic mean of the number of colonies counted in the two dishes.

10.2 Plates containing less than five colonies shall only be taken into account if the other dish contains more than five colonies and if the sum of the colonies in the two dishes is more than 10, thus giving an arithmetic mean of a least five. Plates containing more than 300 colonies shall only be taken into account if the other dish contains less than 300 colonies and if the sum of the colonies in the two dishes does not exceed 600, thus giving an arithmetic mean less than 300.

10.2.1 The results should be rounded as follows:

- a) If the number is less than 100, round it to the nearest multiple of 5;
- b) If the number is greater than 100 and does not end in 5, round it to the nearest multiple of 10;

- c) If the number is greater than 100 and ends in 5, round it to the nearest multiple of 20.

10.2.2 Multiply this value by the reciprocal of the corresponding dilution to obtain the number of micro-organisms per millilitre of semen. Express this result as a number between 1.0 and 9.9 multiplied by 10^x , where x is the appropriate power of 10.

10.3 If plates contain less than five colonies, report the result as follows:

Less than 5f micro-organisms per millilitre, the dilution of the initial suspension being 1/f.

10.4 If the plates contain no colonies, report the result as follows:

Less than 1f micro-organisms per millilitre, the dilution of the initial suspension being 1/f.

11 TEST REPORT

11.1 The test report shall show the method used and the results obtained, indicating clearly the method of expression used. It shall also mention any operating details not specified in this standard, or regarded as optional, together with details of any incident likely to have influenced the results.

11.2 The test report shall include all the information necessary for the complete identification of the sample.

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